Differential effects of rapamycin treatment on tonic and phasic GABAergic inhibition in dentate granule cells after focal brain injury in mice

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Abstract

The cascade of events leading to post-traumatic epilepsy (PTE) after traumatic brain injury (TBI) remains unclear. Altered inhibition in the hippocampal formation and dentate gyrus is a hallmark of several neurological disorders, including TBI and PTE. Inhibitory synaptic signaling in the hippocampus is predominately driven by γ -aminobutyric acid (GABA) neurotransmission, and is prominently mediated by postsynaptic type A GABA receptors (GABA_AR's). Subsets of these receptors involved in tonic inhibition of neuronal membranes serve a fundamental role in maintenance of inhibitory state, and GABA_AR-mediated tonic inhibition is altered functionally in animal models of both TBI and epilepsy. In this study we assessed the effect of mTOR inhibition on hippocampal hilar inhibitory interneuron loss and synaptic and tonic GABAergic inhibition of dentate gyrus granule cells (DGCs) after controlled cortical impact (CCI), to determine if mTOR activation after TBI modulates GABA_AR function. Hilar inhibitory interneuron density was significantly reduced 72 hours after CCI injury in the dorsal two-thirds of the hemisphere ipsilateral to injury compared to the contralateral hemisphere and sham controls. Rapamycin treatment did not alter this reduction in cell density. Synaptic and tonic current measurements made in DGCs at both 1-2 and 8-13 weeks post-injury indicated reduced synaptic inhibition and THIP-induced tonic current density in DGCs ipsilateral to CCI injury at both time points post-injury, with no change in resting tonic GABA_AR-mediated currents. Rapamycin treatment did not alter the reduced synaptic inhibition observed in ipsilateral DGCs 1-2 weeks post-CCI injury, but further reduced synaptic inhibition of ipsilateral DGCs at 8-13 weeks post-injury. The reduction in THIP-induced tonic current after injury, however, was prevented by rapamycin treatment at both time points. Rapamycin treatment thus differentially modifies CCI-induced changes in synaptic and tonic GABA_AR-mediated currents in DGCs.

Keywords:

mTOR, controlled cortical impact, hippocampus, inhibition, dentate gyrus, tonic current, synapse, γ -aminobutyric acid receptors

Introduction

In cases of moderate to severe TBI, the incidence of spontaneous seizure development is ~10 to 30 times higher than those of non-injured patients (Temkin, 2009; Temkin et al., 1998; Temkin et al., 2001). The development of spontaneous seizures after a latent, non-symptomatic post-injury period is referred to as post-traumatic epilepsy (PTE) and often manifests as temporal lobe epilepsy (TLE) (Annegers et al., 1998; Caveness et al., 1979; Diaz-Arrastia et al., 2000; Englander et al., 2003; Hudak et al., 2004). One of the primary origins for seizure generation in patients with TLE is the hippocampus, which is also often affected by TBI (Hall et al., 2005a; Hall et al., 2005b; Newcomb et al., 1997; Saatman et al., 2006). After focal TBI in mice, both excitation and inhibition of dentate granule cells (DGCs) is altered in conjunction with PTE development (Hunt et al., 2009, 2010, 2011). Sprouting of DGC axons into the granule cell and inner molecular layer of the dentate gyrus (i.e. mossy fiber sprouting) leads to recurrent excitation of DGCs and disrupts the normal information processing of the hippocampus. Synaptic inhibition of DGCs is also altered after TBI, and GABA_A receptors (GABA_AR's) undergo functional changes in models of TBI and epilepsy (Boychuk et al., 2016; Hunt et al., 2011; Mchedlishvili et al., 2010; Raible et al., 2012; Raible et al., 2015).

One prominent cell signaling pathway associated with both TBI and epileptogenesis is the mammalian target of rapamycin (mTOR). Some of the common biochemical and cellular structure alterations associated with increased mTOR activation after TBI are increased protein synthesis and phosphorylation, axon sprouting, cell migration, and neurogenesis (Buckmaster and Wen, 2011; Butler et al., 2015; Guo et al., 2013; Heng et al., 2013; Hester and Danzer, 2013; LaSarge et al., 2015). Previous studies of mTOR activation in both TBI and epilepsy models have predominately focused on the excitatory circuitry of the hippocampus and, in particular,

axonal plasticity of DGCs (Buckmaster and Schwartzkroin, 1994; Buckmaster and Wen, 2011; Butler et al., 2015; Guo et al., 2013; Heng et al., 2013; Hester and Danzer, 2013; Hunt et al., 2012; Hunt et al., 2009, 2010; LaSarge et al., 2015; Winokur et al., 2004; Wuarin and Dudek, 1996; Zeng et al., 2008). The effect of mTOR inhibition in TBI and TLE models on neuron loss is controversial (Buckmaster and Wen, 2011; Butler et al., 2015; Guo et al., 2013), but rapamycin treatment suppresses TLE-related morphological changes in hilar inhibitory interneurons (Buckmaster and Wen, 2011). Hilar interneuron loss likely contributes to altered GABAergic circuitry function and receptor responsiveness after TBI (Boychuk et al., 2016; Hunt et al., 2011; Mtchedlishvili et al., 2010), but effects of mTOR modulation on inhibitory signaling in the dentate gyrus after focal brain injury have not been adequately described. We hypothesized that chronic rapamycin treatment following CCI injury leads to reduced TBI-induced changes in synaptic and non-synaptic inhibition of DGCs in the ipsilateral hemisphere.

Methods

Animals: Six-to-eight week old (28-32 g) male CD-1 (Harlan, Indianapolis, IN) or FVB-TgN(GadGFP)45704Swn (i.e. GIN) mice (The Jackson Laboratories, Bar Harbor, ME), which express enhanced green fluorescent protein (eGFP) in the somatostatin-positive subset of cortical GABAergic neurons (Oliva et al., 2000), were housed in the University of Kentucky vivarium on a 14 hr light/10 hr dark cycle. Mice were housed for a minimum of 7 days prior to experimentation and food and water was provided *ad libitum*. All procedures were approved by the University of Kentucky Animal Care and Use Committee and adhered to the NIH guidelines for the care and use of animals.

Traumatic Brain Injury: Mice were subjected to severe unilateral, cortical contusion injury by controlled cortical impact (CCI), as previously described (Hunt et al., 2012; Hunt et al., 2009, 2010, 2011). Briefly, mice were anesthetized by 2% isoflourane inhalation and placed in a stereotaxic frame. The skull was exposed by midline incision, and a ~5 mm diameter craniotomy was made lateral to the sagittal suture and centered between bregma and lambda. The skull cap was removed without damage to the exposed underlying dura. The contusion device consisted of a computer-controlled, pneumatically driven impactor fitted with a beveled stainless-steel tip 3 mm in diameter (TBI-0310; Precision Systems and Instrumentation, Fairfax, VA). Brain injury was delivered using this device to compress the cortex to a depth of 1.0 mm (hard stop) at a velocity of 3.5 m/sec and 500 msec duration. A qualitative postoperative health assessment was performed daily for 4 days after CCI and periodically thereafter.

Rapamycin/ Vehicle injection: Rapamycin (LC Laboratories, Woburn, MA) was initially dissolved in 100% ethanol (20 mg/ml), stored at -20 °C, and diluted in a vehicle solution containing 5% Tween 80, 5% PEG 400, and 4% ethanol in double distilled H₂O immediately before injection, as described previously (Butler et al., 2015; Guo et al., 2013; Heng et al., 2013). Rapamycin (3 mg/kg) or vehicle solution was injected after mice regained consciousness following CCI injury (~20-30 minutes after injury) and continued once daily until the mouse was used for experimentation. All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless otherwise indicated.

Western blot: Western blot was performed with modifications as described previously (Halmos et al., 2015). Protein lysates were generated from whole hippocampi using a lysis buffer

containing: 2 ml 10X Ripa Lysis Buffer (20-188, Millipore), 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, 80 µL of protease inhibitor (P8340, Sigma), 80 µL of phosphatase inhibitor (P5726, Sigma) diluted in ddH₂O (total volume: 20 ml). The Lowry assay was used to determine protein concentration. Extracted protein (30 µg) was loaded into 10% SDS-polyacrylamide gels and run for 1.5 hrs at 155 V and then transferred to nitrocellulose membranes. Membranes were blocked for 2 hours using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and then incubated with rabbit polyclonal antibodies raised against ribosomal S6 phosphorylated at serine 235 (anti-pS6; 1:1000; Cell Signaling Technology, Beverly, MA), or β-actin (1:5000; Abcam, Cambridge, MA) overnight at 4°C in 50% Odyssey Blocking buffer and 50% TBS. Membranes were rinsed 5 times and then incubated with 700 IR goat-anti-rabbit secondary antibody (1:10000; Rockland, Limerick, PA). Membranes were rinsed 5 times and imaged using a LI-COR Odyssey Infrared Imaging System (model 9120, LI-COR Biosciences, Lincoln, NE). Protein bands were quantified using Image J software (NIH, Bethesda, MA).

eGFP+ Hilar Cell Density Measurements: 72 hours after injury, GIN mice were anesthetized by isoflurane inhalation to effect (lack of tail pinch response) and perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH=7.4) while anesthetized. Brains were then removed and placed in fixative overnight, equilibrated in 30% sucrose for 48 hours, and sectioned at 30 µm on a cryostat (-22°C). 1-in-6 serial section series (180 µm between mounted sections) containing the hippocampus were mounted in Vectashield with DAPI counterstain (Vector Labs; Burlingame, CA) and images were taken on a Zeiss 5 Live confocal microscope (Zeiss; Oberkochen, Germany). Hilar area was measured using ImageJ software to trace along the inner surface of the upper and lower blades of the dentate gyrus and in a line from the tip of

each blade of the dentate gyrus to the proximal most point of CA3 pyramidal cell layer. Cell counts from each section were divided by this hilar area measure to give enhanced green fluorescent protein (eGFP) cell density measures for each section. On average, 15 serial sections were taken from each animal and cell density measurements were then averaged for each animal, divided into dorsal, medial, and ventral thirds of hippocampus. Each area covered ~900 μm . The investigator was blinded to animal treatment for all cell counts.

Slice Preparation: Mice were deeply anesthetized by isoflurane inhalation and decapitated while anesthetized. The brain was removed and placed in ice-cold (2-4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 3 KCl, 1.3 CaCl_2 , 26 NaHCO_3 , 1.3 MgCl_2 , 11 glucose and 1.4 NaH_2PO_4 equilibrated with 95% O_2 -5% CO_2 (pH 7.2-7.4). ACSF always contained the ionotropic glutamate receptor antagonist, kynurenic acid (1 mM; Sigma Aldrich, St. Louis, MO). Brains were blocked, glued to a sectioning stage, and slices (350 μm) were cut in the coronal plane into cold, oxygenated ACSF using a vibrating microtome (Vibratome Series 1000; Technical Products International, St. Louis, MO). The hippocampus was then isolated from surrounding tissue, making sure to completely remove the entorhinal cortex. Slices were transferred to a storage chamber containing oxygenated ACSF at 32-34°C. Slices of the dorsal hippocampus from the hemispheres ipsilateral and contralateral to CCI injury were used in these experiments and compared to comparable slices from sham-injured mice (i.e., craniotomy, but no impact injury). Both the hemisphere and dorsal-to-ventral location of each slice was documented; recordings were performed in the dorsal half of the hippocampus (coronal sections), corresponding to areas demonstrating the most cellular changes in previous reports (Butler et al., 2015; Hunt et al., 2012; Hunt et al., 2009).

Whole cell recordings: Coronal hippocampal slices containing the dorsal third of the dentate gyrus were transferred to a recording chamber on an upright, fixed-stage microscope equipped with infrared, differential interference contrast optics (i.e., IR-DIC; Olympus BX50WI), where they were perfused with continuously warmed (32-34°C) ACSF. Recordings were performed from DGCs, which were identified using DIC imaging. Recording pipettes were pulled from borosilicate glass (1.65 mm outer diameter, 0.45 mm inner diameter; King Precision Glass, Claremont, CA) with a P-87 puller (Sutter Instrument, Novato, CA). The intracellular solution contained (in mM): 140 Cs⁺-gluconate, 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 1 CaCl₂, 3 CsOH, and 2 ATP; pH=7.15-7.3. Open tip series resistance was 2-5 MΩ. Recordings were obtained using an Axon Axopatch 200B (Molecular Devices, Sunnyvale, CA), low-pass filtered at 6 kHz, digitized at 20 kHz with a 1322A Digidata (Molecular Devices), and acquired using pClamp 10.2 (Clampfit, Molecular Devices, Sunnyvale, CA). Cells were voltage-clamped at 0 mV for 5-10 min to allow equilibration of pipette and intracellular solutions prior to data collection, after which time whole-cell patch-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) were obtained. An initial 5 minute baseline recording period was followed by the addition of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP; gaboxadol; 3 μM; Sigma-Aldrich) to the ACSF, a super-agonist of GABA_AR's containing δ subunits. At the concentration used here (3 μM), THIP selectively targets δ subunit-containing, putatively extrasynaptic GABA_ARs that contribute to the tonic GABA current (Brown et al., 2002; Ebert et al., 1994). THIP was applied for 10 minutes and followed by addition of bicuculline methiodide (30 μM; Tocris Bioscience; Minneapolis, MN) to the ACSF for 8 minutes to eliminate postsynaptic inhibitory currents and measure resting tonic current.

Recordings exhibiting $>25 \text{ M}\Omega$ (mean= $13.53 \pm 0.62 \text{ M}\Omega$, $n=88$) series resistance or in which series resistance changed by $>20\%$ were discarded.

Data and statistical analysis: Data analysis was performed using MiniAnalysis 6.0.3

(Synaptosoft; Decatur, GA) and Graphpad (La Jolla, CA) software programs. To measure sIPSC frequency, amplitude, and kinetics, a 3 minute sample of the baseline recording was used. Only events with three times the amplitude of root mean squared (RMS) baseline noise were included (RMS= 1.59 ± 0.05 , $n=88$). Events characterized by a typical fast rise phase and exponential decay were automatically detected and then manually verified in MiniAnalysis. Tonic current amplitude was detected by subtracting mean steady-state holding current values during baseline or during THIP application from mean steady-state holding current values in the presence of bicuculline methiodide, a competitive antagonist of GABA_AR 's. Tonic current amplitude values were normalized to whole cell capacitance (Glykys and Mody, 2007). For hilar interneuron counts, groups were compared using a Two Way ANOVA followed by Bonferroni's *post hoc* test at each septo-temporal hippocampal portion measured. For all electrophysiological data, groups were compared using a One Way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean $\pm$ SEM.

Results

pS6 Expression: After brain injury the ipsilateral hippocampus exhibits increased pS6 expression that indicates elevated mTOR signaling and rapamycin treatment restores this expression to control levels (Guo et al., 2013). Western blot analysis was used to confirm the effect of

rapamycin treatment on pS6 expression here by examining hippocampi contralateral (control) and ipsilateral (injured) to CCI injury with vehicle treatment, and ipsilateral hippocampus of CCI injured mice with rapamycin treatment (Figure 1). The ipsilateral hippocampus of CCI injured mice with vehicle treatment exhibited an increase in pS6 expression compared to contralateral controls (Control: 0.16 ± 0.03 a.u., CCI ipsi: 0.39 ± 0.08 a.u.; $p=0.0004$, $F(2,14)=14.80$; One Way ANOVA, Tukey's, $p=0.0008$ vs control). Rapamycin treatment after CCI injury reduced pS6 expression in the ipsilateral hippocampus compared to CCI injured mice given vehicle treatment (CCI+Rapa: 0.09 ± 0.003 a.u.; Tukey's, $p=0.0007$ vs CCI ipsi) to levels similar to that in controls (Tukey's, $p=0.40$). Rapamycin treatment therefore prevented the increase in pS6 expression after CCI injury.

eGFP+ Hilar Interneuron Counts: After brain injury, a significant percentage of hilar GABA neurons are depleted (Lowenstein et al., 1992; Santhakumar et al., 2000; Toth et al., 1997). Cell loss following CCI injury peaks within the first three days after insult (Anderson et al., 2005). Therefore, we measured eGFP+ cell density at 72 hours post-injury to determine the effect of rapamycin on hilar inhibitory interneuron survival after CCI. Cell density measurements were made in mice from groups of sham operated controls ($n=7$ mice), CCI injured with vehicle treatment ($n=7$ mice), and CCI injured with rapamycin treatment ($n=10$ mice). Figure 2A shows representative images of eGFP+ hilar interneurons with DAPI counterstain in the dorsal, medial, and ventral thirds of the hippocampus from sham injured mice and in the hippocampus ipsilateral to CCI injury with vehicle or rapamycin treatment. CCI injured mice with vehicle or rapamycin treatment exhibited lower eGFP+ hilar cell density compared to sham-injured controls and hemispheres contralateral to injury in the dorsal ($p=0.0033$; $F(5,40)=20.12$; Figure 2B) and

medial ($p=0.0005$; $F(5,40)=10.57$; Figure 2B) portions of the hippocampus (Table 1). In the ventral portions of hippocampus there was no significant difference between experimental groups ($p>0.05$; Table 1; Figure 2B). Rapamycin treatment did not alter hilar interneuron survival after focal brain injury in the dorsal or medial portions of the hippocampus (Bonferroni, $p>0.05$). These data indicate that rapamycin does not affect the loss of eGFP⁺ hilar interneurons ipsilateral to CCI injury.

sIPSC frequency, amplitude, and kinetics in DGCs: Although it has been shown that rapamycin reduces axon sprouting of eGFP⁺ hilar inhibitory interneurons after pilocarpine treatment in GIN mice (Buckmaster and Wen, 2011), it is not known if this is accompanied by altered inhibition of DGCs. sIPSC frequency was measured in DGCs from sham-injured mice, CCI-injured mice with vehicle treatment, and CCI-injured mice with rapamycin treatment at 1-2 (sham= 5 mice, CCI= 7 mice, CCI+Rapa= 8 mice; Figure 3A) or 8-13 weeks after injury (sham= 6 mice, CCI= 8 mice, CCI+Rapa= 7 mice; Figure 3C). At 1-2 weeks post-injury, there was no difference in sIPSC frequency between groups in DGCs contralateral to injury (Sham contra: 2.43 ± 0.22 Hz, $n=5$ cells, CCI contra: 2.41 ± 0.23 Hz, $n=9$ cells, CCI+Rapa contra: 2.29 ± 0.27 Hz, $n=12$ cells; $F(2,24)=0.1112$, $p=0.8953$; One Way ANOVA, Tukey's; Figure 3B). DGCs from the ipsilateral hemisphere of CCI injured mice with either vehicle or rapamycin treatment exhibited reduced sIPSC frequency relative to DGCs from the ipsilateral hemisphere of sham injured mice (Sham ipsi: 2.61 ± 0.22 Hz, $n=6$ cells, CCI ipsi: 1.44 ± 0.09 Hz, $n=9$ cells, CCI+Rapa ipsi: 1.39 ± 0.26 Hz, $n=13$ cells; $F(2,27)=4.112$, $p=0.0184$; One Way ANOVA, Tukey's; Figure 3B), but there was no difference between ipsilateral DGCs from vehicle- and rapamycin-treated mice after CCI injury ($p=0.8775$). Rapamycin treatment therefore did not alter the reduction in sIPSC frequency detected in DGCs ipsilateral to CCI injury.

As at the earlier time point post-injury, there was no difference in sIPSC frequency between groups in the contralateral hemisphere 8-13 weeks after injury (Sham: 2.24 ± 0.14 Hz, $n=5$ cells, CCI contra: 2.13 ± 0.25 Hz, $n=8$ cells, CCI+Rapa contra: 1.71 ± 0.38 Hz, $n=8$ cells; $F(2,19)=0.7659$, $p=0.4803$; One Way ANOVA, Tukey's; Figure 3D). In CCI-injured mice, DGCs from the ipsilateral hemisphere of vehicle and rapamycin treatment groups exhibited reduced sIPSC frequency compared to DGCs from sham-treated controls 8-13 weeks post-injury, which was similar to the earlier time point (Sham ipsi: 2.10 ± 0.10 Hz, $n=5$ cells, CCI ipsi: 1.35 ± 0.11 Hz, $n=7$ cells, CCI+Rapa ipsi: 0.83 ± 0.15 Hz, $n=8$ cells; $p<0.0001$; $F(2,17)=20.38$; One Way ANOVA, Tukey's; Figure 3D). Unlike at 1-2 weeks post-injury, there was a significant reduction in sIPSC frequency 8-13 weeks after injury in DGCs from the ipsilateral hemisphere of rapamycin treated mice compared to the vehicle-treated CCI-injured mice ($p=0.017$). These data indicate that reduced synaptic inhibition in the ipsilateral hemisphere observed 1-2 weeks after CCI was not altered by rapamycin treatment. However, continued rapamycin treatment for 8-13 weeks further reduced synaptic inhibition of DGCs ipsilateral to CCI injury.

The kinetics of sIPSCs were also compared between sham-injured mice, CCI-injured mice with vehicle treatment, and CCI-injured mice with rapamycin treatment. Rapamycin had no effect on sIPSC decay time constant or rise time relative to CCI-injured mice given vehicle at either time point following injury. Additionally, sIPSC amplitude was not different in DGCs from CCI-injured mice given rapamycin treatment relative to the other groups 1-2 weeks post-injury. At 8-13 weeks post-injury, however, ipsilateral DGCs from CCI-injured mice given rapamycin treatment exhibited reduced sIPSC amplitude compared to ipsilateral DGCs from vehicle-treated CCI injured mice and a trend toward reduced sIPSC amplitude relative to

ipsilateral DGCs from sham controls (sham ipsi: 20.36 ± 2.02 , CCI + vehicle ipsi: 21.42 ± 2.10 , CCI+Rapa ipsi: 14.87 ± 1.32 ; $p=0.0229$; $F(2,17)=4.547$; One Way ANOVA, Tukey's, $p=0.03$ CCI + vehicle ipsi vs CCI+Rapa ipsi; $p=0.08$ sham ipsi vs CCI+Rapa ipsi). No changes in amplitude were detected in DGCs from the contralateral hemispheres 8-13 weeks post-injury. Collectively, the changes in sIPSC frequency and amplitude in ipsilateral DGCs from CCI-injured mice given rapamycin treatment at 8-13 weeks post-injury suggest both pre- and post-synaptic effects of rapamycin treatment on inhibitory circuitry after CCI injury.

Tonic GABA_AR currents 1-2 week post-injury: THIP-induced tonic GABA_AR-mediated currents in DGCs are altered in rodent models of TBI and TLE (Boychuk et al., 2016; Gupta et al., 2012; Mchedlishvili et al., 2010; Pavlov et al., 2011). In mice, resting tonic GABA_AR current is not altered after CCI, but the THIP- or neurosteroid-induced tonic GABA_AR current amplitude is reduced in the ipsilateral hemisphere after CCI, and this reduction is sustained for at least 3 months (Boychuk et al., 2016). Although synaptic GABAergic transmission was not altered by rapamycin treatment 1-2 weeks after injury, it is not known what effect rapamycin has on resting or THIP-induced tonic GABA_AR mediated currents. Therefore, both resting and THIP-induced GABA_AR mediated tonic currents were measured 1-2 weeks post injury in sham and vehicle- or rapamycin-treated mice after CCI.

Figure 4A shows representative traces of whole-cell recordings of DGCs from sham-injured mice, and contralateral and ipsilateral DGCs from CCI-injured mice with vehicle or rapamycin treatment. Rapamycin treatment after CCI did not alter resting tonic GABA_AR-mediated currents in DGCs from either the contralateral (Table 2; $p=0.6772$; $F(2,21)=0.3979$; One Way ANOVA, Tukey's) or ipsilateral hemisphere (Table 2; $p=0.5458$; $F(2,22)=0.6242$; One Way ANOVA, Tukey's; Figure 4A, B). Additionally, there was no change in THIP-induced

tonic GABA current response of DGCs in the contralateral hemisphere of any treatment group (sham contra: 2.98 ± 0.57 pA/mF, n=5 cells, CCI contra: 3.11 ± 0.50 pA/mF, n=9 cells, CCI+Rapa contra: 3.09 ± 0.44 pA/mF, n=8 cells; $p=0.9862$; $F(2,21)=0.01388$; One Way ANOVA, Tukey's; Figure 4C). Similar to our previous report (Boychuk et al., 2016), CCI injury resulted in a reduction in the response to THIP in DGCs ipsilateral to injury relative to DGCs ipsilateral to sham-operated controls (sham ipsi: 3.08 ± 0.22 pA/mF, n=6 cells, CCI ipsi: 1.81 ± 0.30 pA/mF, n=9 cells; $p=0.0443$; $F(2,26)=3.560$; One Way ANOVA, Tukey's; Figure 4C). Rapamycin treatment prevented the reduction of THIP-induced tonic GABA_AR current amplitude in ipsilateral DGCs 1-2 weeks after injury (CCI+Rapa ipsi: 3.22 ± 0.56 pA/mF, n=10 cells, $p=0.8402$ relative to sham, $p=0.0321$ relative to CCI+vehicle ipsi; Figure 4C). These data indicated that rapamycin treatment did not alter resting tonic GABA_AR currents following CCI injury, but eliminated the reduction in THIP-induced GABA_AR current amplitude measured in DGCs ipsilateral to CCI injury.

Tonic GABA_AR currents 8-13 weeks post-injury: Based on reduced synaptic GABAergic transmission to ipsilateral DGCs 8-13 weeks after injury and attenuated THIP-induced tonic GABA_AR currents 1-2 weeks after CCI injury, we tested the effect of chronic rapamycin treatment 8-13 weeks after CCI on resting and THIP-induced GABA_AR-mediated tonic currents. Figure 4A shows representative traces of whole-cell recordings of DGCs from sham, and contralateral and ipsilateral to CCI injury with vehicle or rapamycin treatment. There was no difference in THIP-induced tonic current amplitude in DGCs from the contralateral hemisphere of any treatment group (sham contra: 3.43 ± 0.33 pA/mF, n=5 cells, CCI contra: 3.79 ± 0.39 pA/mF, n=8 cells, CCI+Rapa contra: 3.34 ± 0.38 pA/mF, n=8 cells; $p=0.6191$; $F(2,22)=0.4912$;

One Way ANOVA, Tukey's; Figure 5C). Similar to a previous report (Boychuk et al., 2016), and similar to currents measured 1-2 weeks post-injury, DGCs from the ipsilateral hemisphere of CCI injured mice exhibited reduced THIP-induced tonic currents compared to DGCs from the ipsilateral hemisphere of sham-operated controls (sham ipsi: 3.63 ± 0.37 pA/mF, n=5 cells, CCI ipsi: 1.69 ± 0.23 pA/mF, n=7 cells; $p=0.0008$; $F(2,21)=10.69$; One Way ANOVA, Tukey's; Figure 5C). Chronic rapamycin treatment for 8-13 weeks after CCI attenuated the reduction in THIP-induced tonic currents in the ipsilateral hemisphere (CCI+Rapa ipsi: 2.84 ± 0.26 pA/mF, n=8 cells; $p=0.0982$ relative to sham, $p=0.0064$ relative to CCI+vehicle ipsi). There was no difference in resting GABA_AR-mediated tonic current in DGCs from any treatment group contralateral (Table 2; $p=0.9103$; $F(2,22)=0.09441$; One Way ANOVA, Tukey's), or ipsilateral to the injury (Table 2; $p=0.9244$; $F(2,21)=0.07894$; One Way ANOVA, Tukey's; Figure 5B). Although resting tonic current was unaltered after CCI or by rapamycin treatment post-injury, THIP-induced tonic current amplitude was reduced ipsilateral to CCI-injury. Rapamycin treatment attenuated this reduction and restored tonic inhibition to values similar to controls. This occurred despite diminished synaptic inhibition in rapamycin treated mice.

Discussion

Little is known about the effects of rapamycin treatment on GABAergic signaling in the dentate gyrus after CCI or in models of epilepsy. Most previous work on the role of mTOR modulation in epilepsy and in models of TBI has focused on plasticity of excitatory circuitry, including mossy fiber sprouting and synaptic reorganization of glutamatergic circuits in the dentate gyrus (Buckmaster et al., 2009; Butler et al., 2015; Guo et al., 2013; Heng et al., 2013; Zeng et al.,

2009). Previous studies testing rapamycin's effects on excitatory circuitry observed a reemergence of seizures and mossy fiber sprouting when treatment was removed a few weeks after brain insult (Buckmaster et al., 2009; Guo et al., 2013). Additionally, chronic mTOR inhibition in patients with cancer can lead to the eventual upregulation of ERK/MAPK/Akt pathways in the cancerous tumors (O'Reilly et al., 2006; Tabernero et al., 2008), which can upregulate mTOR complex 2 and compensate for the rapamycin-induced suppression of mTOR complex 1. Analogous compensation may be a consideration in chronic rapamycin treatment for patients with epilepsy. Rapamycin treatment was therefore maintained throughout the duration of these experiments in order to better understand the cellular effects of long-term, continual rapamycin treatment after CCI injury.

Although effects of rapamycin treatment on hilar inhibitory neuron morphology have been reported (Buckmaster and Wen, 2011), mTOR's role in regulating inhibitory synaptic reorganization or tonic GABA currents in DGCs has not been previously studied. However, axon sprouting in surviving inhibitory hilar interneurons could be a mechanism to compensate for the loss of GABAergic neurons and their connections to DGCs after pilocarpine-induced status epilepticus or TBI. Alternatively, aberrant axon sprouting could be a generalized response of injured neurons. Here, chronic rapamycin treatment did not prevent hilar inhibitory neuron cell loss after CCI, and suppressed sIPSC frequency and amplitude to a greater extent than CCI alone by 8-13 weeks post-injury. This is consistent with reduced axon sprouting in eGFP hilar interneurons after rapamycin treatment in mice with acquired TLE (Buckmaster and Wen, 2011), and could be predicted to further diminish synaptic inhibition of DGCs. The CCI protocol used here has previously been shown to result in development of spontaneous seizures in approximately 40-50% of mice several weeks post-injury (Butler et al., 2015; Guo et al., 2013;

Hunt et al., 2009). Seizures were not measured in the present study because no separate grouping or multimodal populations matching these seizure expression percentages were observed for any data reported here. As a result, we did not further test the relationship between these changes and seizure expression for any of the outcome measures. Reduced synaptic inhibition after rapamycin treatment post-CCI would be inconsistent with an antiepileptogenic effect of the treatment. This feature would actually be predicted to increase seizure susceptibility by further aggravating the imbalance of excitation and inhibition of DGCs in the ipsilateral hemisphere, which could be further exacerbated after cessation of treatment and re-initiation of the epileptogenic process.

Synaptic inhibition of DGCs is only one form of inhibitory control, and small changes in tonic GABA_AR mediated currents may have a large impact on inhibitory control of DGCs (Coulter and Carlson, 2007; Mody and Pearce, 2004). Unlike effects on synaptic inhibition, rapamycin attenuated the reduction in THIP-sensitivity seen in DGCs ipsilateral to injury at both early and chronic phases post-injury. Present and previously reported results indicate that, while resting tonic current is unaltered by injury or rapamycin treatment, the effect on THIP sensitivity was responsive to mTOR inhibition. A previous study concluded that receptor trafficking was affected after CCI to account for the diminished THIP-sensitivity in DGCs ipsilateral to injury (Boychuk et al., 2016). This normalized response suggests a role for mTOR in receptor trafficking, likely targeting the available, unbound receptors in the ipsilateral hemisphere following CCI injury. This could also indicate a change in either the GABA_AR subtypes responding to different stimuli, a reorganization of the pentameric archetypal structure of the GABA_AR's, such as the substitution of different α subunits or δ/γ subunits in disease states, or a change in receptor distribution within the membrane.

The hilar inhibitory interneuron loss observed here is consistent with previous work showing that CCI injury results in loss of hilar neurons (Hicks et al., 1993; Lowenstein et al., 1992). Following CCI, higher doses of rapamycin treatment resulted in a reduction in Fluoro-Jade B staining in a limited area of the hippocampus ~1mm posterior to injury epicenter (Butler et al., 2015; Guo et al., 2013), although cell loss was still significant relative to uninjured-controls. The loss of eGFP+ interneurons after CCI in this study was observed only in the dorsal and middle hippocampal regions along the septo-temporal axis after CCI injury, with no measurable cell loss occurring in the ventral third of the hippocampus. Rapamycin had no effect on this inhibitory hilar interneuron loss, consistent with previous conclusions (Butler et al., 2015). Furthermore, a lack of rapamycin treatment effect on cell loss in the dorsal and middle regions of hippocampus after CCI suggests effects on synaptic or tonic inhibition in DGCs ipsilateral to injury are not likely to be related to prevention of eGFP+ interneuron loss, though it does not rule out effects on other interneuron phenotypes.

Although rapamycin treatment had little effect on the reduction in sIPSC frequency ipsilateral to CCI injury 1-2 weeks after injury, continued administration of rapamycin for 8-13 weeks post-injury further reduced sIPSC frequency in ipsilateral DGCs relative to vehicle treatment. This result is consistent with reduced axon sprouting of hilar interneurons in pilocarpine treated mice that received rapamycin (Buckmaster and Wen, 2011), and suggests the reduced axon sprouting after rapamycin treatment may also contribute to reduced sIPSC frequency in DGCs ipsilateral to CCI. Similarly, in models of both TLE and TBI surviving hilar inhibitory interneurons have been shown to receive increased excitatory input and exhibit increased action potential activity (Halabisky et al., 2010; Hunt et al., 2011). This increase in excitatory synaptic drive is due to axon plasticity of both DGCs (i.e. mossy fiber sprouting) and

CA3 pyramidal neurons (i.e. CA3 back projections). Rapamycin treatment in models of both TLE and TBI reduces mossy fiber sprouting, and could also lead to reduced excitatory drive to this interneuron population, contributing to reduced recurrent synaptic inhibition of DGCs (Buckmaster et al., 2009; Buckmaster and Lew, 2011; Butler et al., 2015; Guo et al., 2013; Heng et al., 2013; Zeng et al., 2009). Effects of rapamycin treatment on synaptic inhibition may reflect a role of mTOR in functional morphological changes underlying synaptic reorganization after focal brain injury. In addition to these possible presynaptic actions of rapamycin treatment, the drug also reduced sIPSC amplitude in CCI-injured mice at 8-13 weeks post-injury. This is consistent with results from a previous report of mTOR modulation of miniature IPSCs in a cell culture system (Weston et al., 2012), and further suggests rapamycin has both pre- and postsynaptic effects on inhibitory circuitry of the dentate gyrus after CCI injury.

The processes underlying altered GABA_AR function continue to be inadequately understood, especially in models of epilepsy and TBI. The precise mechanisms by which cellular changes in GABA_AR-mediated responses occur remain unknown and alterations of both GABA_AR location and organization are not consistently reported across various models of epilepsy and TBI. This variation may reflect differing sampling techniques, brain area selection, and the various mechanisms by which these animal models achieve epileptogenesis. One of the more interesting differences in modulation of GABA_AR's between the models of TBI used to study PTE is the reversion of changes in GABA_AR's after a latent period in the fluid percussion injury model (Pavlov et al., 2011), which is not seen in the CCI model. This difference may reflect the different mechanisms which underscore the increased susceptibility of epileptogenesis in mice receiving CCI injury vs. fluid percussion injury. Although some work in cell culture has linked the Akt→mTOR pathway to altered GABA_AR phosphorylation and surface expression

(Wang et al., 2003), our understanding of how this translates to normal function and in models of disease is insufficient.

mTOR's role in cortical excitation after TBI and in severe forms of epilepsy has been investigated rigorously, but the role it plays in regulating inhibition is less clear. This study suggests inhibition of mTOR activity following CCI injury could promote maintenance of normal responses to THIP-induced GABA_AR activity, but that long term rapamycin treatment may also lead to reduced synaptic inhibition of DGCs. Effects of mTOR inhibition on epileptogenesis do not seem to outlast treatment (Buckmaster et al., 2009; Guo et al., 2013), possibly due to persistent effects of injury-induced interneuron loss or effects of rapamycin on potentially compensatory axon sprouting and synaptic reorganization of inhibitory circuits. This detriment could contribute to the eventual development of an epileptogenic circuit and recurrence of seizure susceptibility after treatment removal. Prolonged rapamycin treatment could therefore exacerbate the imbalance of excitation and inhibition in the dentate gyrus after TBI, and could potentially lead to the re-activation of cell signaling pathways involving the mTOR complex 2. These potential pitfalls and the mechanisms by which mTOR contributes to GABA_AR modulation require further investigation, which would not only be informative but critical to our understanding of the complex nature of how changes in inhibitory synaptic organization and the GABA_AR system relate to the development of PTE.

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Author contributions

JAB, CRB, and BNS contributed to the conception and design of the work; acquisition and analysis were performed by JAB and CRB; interpretation of the data was performed by JAB, CRB, and BNS. JAB, CRB and BNS drafted and revised manuscript and approve of the version to be published.

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Figures

Figure 1: Phosphorylated S6 (pS6) expression 24 hours after CCI injury. A. Representative Western blot image of pS6 and β -actin expression in hippocampal homogenates 24 hours post-injury from three different groups: contralateral controls (Control), ipsilateral to CCI injury + vehicle (CCI Ipsi), and ipsilateral to CCI injury + 3 mg/kg rapamycin (CCI+Rapa Ipsi). B. Graph of mean normalized pS6 expression 24 hours post-injury (arbitrary units, a.u.). The hippocampus from CCI Ipsi mice exhibited increased pS6 expression compared to contralateral (control) hippocampus; rapamycin treatment reduced pS6 expression to control levels after this injury. Error bars indicate SEM; asterisk (*) indicates $p < 0.05$.

Figure 2: eGFP+ cell density 72 hours post-CCI injury. A. Representative images of eGFP+ cell distribution in the hilus 72 hours post-injury in three different groups: ipsilateral to sham injury (Sham), ipsilateral to CCI injury + vehicle (CCI Ipsi), and ipsilateral to CCI injury + rapamycin at 3 mg/kg (CCI+Rapa Ipsi) in the dorsal, medial, and ventral thirds of hippocampus. B. Representative histograms of mean eGFP+ cell density 72 hours post-injury in ipsilateral to sham injury, CCI injury with vehicle treatment, and CCI injury with rapamycin treatment in the dorsal, medial and ventral thirds of hippocampus. In the dorsal and medial thirds, sections ipsilateral to CCI injury exhibited decreased eGFP+ cell density compared to sham and contralateral hemispheres. Rapamycin treatment did not alter the reduction in eGFP+ cell density. No difference was observed between any experimental group in the ventral third of hippocampus. Error bars indicate SEM; asterisk (*) indicates $p < 0.05$ versus sham.

Figure 3: sIPSCs in dentate granule cells (DGCs) at 1-2 and 8-13 weeks post-injury.

Representative traces showing sIPSCs in DGCs 1-2 weeks post-injury in five different groups:

sham-operated control, contralateral to CCI injury + vehicle (CCI Contra), ipsilateral to CCI injury + vehicle (CCI Ipsi), contralateral to CCI injury + rapamycin at 3 mg/kg (CCI+Rapa Contra), and ipsilateral to CCI injury + rapamycin at 3 mg/kg (CCI+Rapa Ipsi). B.

Representative histograms of mean sIPSC frequency 1-2 weeks post-injury. DGCs in the ipsilateral hemispheres exhibited decreased sIPSC frequency after CCI relative to sham injury.

No significant difference was observed contralateral to injury. C. Representative traces showing sIPSCs in DGCs 8-13 weeks post-injury in the same groups. D. Representative histograms of mean sIPSC frequency 8-13 weeks post-injury. Decreased sIPSC frequency was observed ipsilateral to CCI injury, regardless of treatment. Additionally, CCI+Rapa Ipsi exhibited reduced sIPSC frequency relative to CCI Ipsi. No significant difference was observed in cells from contralateral hemispheres after CCI injury. Error bars indicate SEM; asterisk (*) indicates $p < 0.05$ relative to sham; (#) indicates $p < 0.05$ relative to both sham and CCI Ipsi.

Figure 4: Bicuculline- and THIP-sensitive tonic currents in DGCs 1-2 weeks post-injury.

A. Representative traces from DGCs 1-2 weeks post-injury in five experimental groups: sham-operated control (Sham), contralateral to CCI injury + vehicle (CCI Contra), ipsilateral to CCI injury + vehicle (CCI Ipsi), contralateral to CCI injury + rapamycin at 3 mg/kg (CCI+Rapa Contra), and ipsilateral to CCI injury + rapamycin at 3 mg/kg (CCI+Rapa Ipsi). Cells were voltage-clamped at 0 mV; bars under the Sham trace indicate times of THIP and bicuculline application during recordings. B. Mean bicuculline-sensitive tonic current density 1-2 weeks post-injury in each experimental group. No significant difference was observed for bicuculline

sensitive resting currents after injury between any experimental group. C. Mean THIP-sensitive tonic current density in DGCs 1-2 weeks post-injury. Error bars indicate SEM; asterisk (*) indicates $p < 0.05$ relative to sham.

Figure 5: Bicuculline- and THIP-sensitive tonic currents in DGCs 8-13 weeks post-injury:

A. Representative traces from DGCs 8-13 weeks post-injury in five different experimental groups. Cells were voltage-clamped at 0 mV and bars under Sham trace indicate THIP and bicuculline application during recordings. B. Mean bicuculline-sensitive current density 8-13 weeks post-injury. C. Mean THIP-sensitive current density DGCs 8-13 weeks post-injury. Error bars indicate SEM; asterisk (*) indicates $p < 0.05$ relative to Sham.

Table 1: Inhibitory hilar interneuron cell counts

Experimental group	Dorsal (cells/mm ²)	Medial (cells/mm ²)	Ventral (cells/mm ²)
Sham contra	17.43 ±2.47 n=7	27.77 ±5.34	39.89 ±7.16
Sham ipsi	15.92 ±1.26 n=7	24.21 ±7.53	42.23 ±7.89
CCI contra	12.34 ±3.37 n=7	22.66 ±3.72	34.15 ±5.37
CCI ipsi	3.16 ±0.67 * n=7	6.61 ±1.63 *	29.28 ±7.14
CCI+Rapa contra	13.02 ±3.41 n=10	28.62 ±5.71	35.89 ±5.72
CCI+Rapa ipsi	5.13 ±0.85 * n=10	7.24 ±2.32 *	36.65 ±3.16

Table 2: Resting tonic GABA currents in DGCs

Animal Group	1-2 weeks post injury resting tonic current (pA/mF)	8-13 weeks post-injury resting tonic current (pA/mF)
Sham Contra	0.64 ±0.17, n=5	0.76 ±0.35, n=5
Sham Ipsi	0.84 ±0.33, n=6	0.79 ±0.34, n=5
CCI Contra	0.84 ±0.13, n=9	0.89 ±0.14, n=8
CCI Ipsi	0.72 ±0.15, n=9	0.74 ±0.13, n=7
CCI+Rapa Contra	0.82 ±0.22, n=8	0.94 ±0.29, n=8
CCI+Rapa Ipsi	0.92 ±0.14, n=10	0.85 ±0.16, n=8

Figure's

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5